BIOTECNOLOGIA VEGETAL COMO HERRAMIENTA EN LA HORTICULTURA ORNAMENTAL

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RESUMEN. La Biotecnología Vegetal o "Cultivo in vitro" de plantas superiores se define como el cultivo y manipulación de plantas, embriones, órganos, explantes, tejidos, células y protoplastos, en un medio nutritivo y en condiciones estériles. La Biotecnología Vegetal ha mostrado un desarrollo espectacular desde 1975, producto de la producción y regeneración de individuos visibles de muchas especies. Además, desde 1980, ha habido una explosión en el desarrollo de técnicas de manipulación genética.

El cultivo *in vitro* de plantas superiores se ha convertido en una importante herramienta, particularmente en la horticultura ornamental y el mejoramiento genético. Las aplicaciones pueden resumirse en los siguientes: cultivo de embriones, germinación de semillas de orquídeas, clonación de orquídeas, producción de plantas libres de enfermedades, micropropagación de muchas especies, variación somaclonal, fertilización *in vitro*, inducción de haploides, manipulación genética, transformación y varias aplicaciones misceláneas (inducción de mutaciones, separación de quimeras, aislamiento de mutantes, duplicación de cromosomas, almacenamiento de plantas a temperaturas bajas, biosíntesis de compuestos, etc.).

La micropropagación ha sido la aplicación más importante. La idea de esta técnica es la de crear posibilidades reales, confiables y seguras, para clonar plantas superiores de una manera libre de enfermedades, rapida y barata comparada con las técnicas *in vivo*. La micropropagación ha desarrollado una gran industria comercial en todo el mundo. Esto se puede ilustrar por el hecho de que, por ejemplo, en 1990, en Holanda existían 80 laboratorios comerciales con una producción de 100 millones de plantas. Este artículo contiene descripciones de los diferentes sistemas de clonación: cultivo de nudos de tallo, brotación múltiple, regeneración de órganos a partir de tejidos diferenciados y regeneración de plantas a partir de tejidos indiferenciados, células o protoplastos. Un prerequisito esencial para una micropropagación exitosa para evitar la variación genética. No se debe olvidar que la micropropagación es una herramienta esencial para la ingeniería genética, la cual sería imposible si no hubiera métodos disponibles de regeneración de plantas a partir de tejidos, células o protoplastos. Problemas durante la micropropagación *en* varios países se ubican en cuatro categorías: técnicas, organización, economía y comercialización. Esos problemas junto con las demandas de consumidores y obreros, deberán ser considerados. Deberá ponerse atención especial a las demandas de los países de Europa Oriental, finalmente, se describen algunos adelantos en micropropagación, enraizamiento de microesquejes *ex-vitro* en sustratos sintéticos inhertes, nuevos sistemas de automatización, el papel oscuro del agar como sustrato y el uso de cápsulas de membrana en lugar de envases de vidrio o plástico.

PALABRAS CLAVE: Micropropagación, orquídeas, sistemas nuevos.

PLANT BIOTECHNOLOGY AS A TOOL IN ORNAMENTAL HORTICULTURE

SUMMARY. Plant biotechnology, or "*in vitro* culture of higher plants", is defined as the culture and manipulation of plants, embryos, organs, explants, tissues, cells and protoplasts on nutrient media under sterile conditions. Plant biotechnology has shown a spectacular development since 1975, resulting in the production and regeneration of viable individuals of many plant species. In addition, since 1980 there has been an explosion in the development of genetic manipulation techniques.

In vitro culture of higher plants has become an important tool, particularly in ornamental horticulture and plant breeding. The applications can be summarized as follows: embryo culture, germination of orchid seeds, cloning of orchids, production of disease-free plants, micropropagation of many plant species, somaclonal variation, *in vitro* fertilization, induction of haploids, genetic manipulation, transformation, and a number of miscellaneous applications (e.g. mutation induction, separation of chimaeras, isolation of mutations, chromosome doubling, storage of plants at low temperatures, biosynthesis of chemicals, etc.).

Micropropagation has become the most important application. The aim of this technique is to create reliable possibilities to clone higher plants in a disease-free, faster and less expensive way as compared with the *in vivo* techniques. Micropropagation has developed intro a large commercial industry all over the world. This can be illustrated by the fact that e.g. in 1990 in The Netherlands 80 commercial laboratories existed with a production of 100 million plants. This lecture contains descriptions of the various

cloning systems; single-node culture, axillary branching; regeneration of organs from differentiated tissues, and regeneration of plants from undifferentiated tissues, cells or protoplasts. An essential prerequisite for successful micropropagation is avoiding genetic variation. One should not forget that micropropagation is an essential tool for genetic engineering, which would be impossible if methods for regeneration of plants from tissues, cells and protoplasm into plants were not available.

Problems encountered in micropropagation in many countries concern four categories: technique, organization, economy, and marketing. These categories together with the demands of customers and labour will be summarized. Special attention will be paid to the demands of the West European countries.

Finally some new developments in micropropagation will be summarized: rooting of microcuttings in inert synthetic supports *ex-vitro*, new automation systems, the obscure role of agar as substrate, and the use of membrane capsules instead of glass or plastic containers.

KEY WORDS: micropropagation, new systems.

1. INTRODUCTION

In vitro culture of higher plants, or plant biotechnology, can be defined as the culture and manipulation of plants, seeds, embryos, organs, explants, tissues, cells and protoplasts on nutrient media under sterile conditions. This technique has shown a spectacular development since 1975. In scientific laboratories various in vitro culture methods have been developed, resulting in the production and regeneration of viable plants. Since 1980 there has been an explosion in the development of genetic manipulation and biotechnology techniques. In vitro culture techniques can be characterized as follows:

1. They occur on micro-scale with small parts of plants i.e. on a relatively small surface area.

2. Micro-organisms as well as other pests are excluded.

3. The enviromental conditions are strongly optimized and well defined with regard to physical as well as nutritional and hormonal factors.

4. They often enable manipulations which previously were very difficult or impossible.

5. The most important aim is often the production of viable plants.

This article contains descriptions of the various types of in vitro culture and their applications with special attention to the propagation and breeding of ornamental crops. A few articles and a new hadbook on in vitro culture (Pierik, 1987, 1988, 1990, 1990e, 1991, 1991b, 1993) have been used as key references to compose this article, for more details and literature the readers are referred to these articles and handbooks. Micropropagation will be dealt with in detail because this is the most important application.

2. APPLICATIONS

Data on application were earlier published (Pierik, 1990e).

2.1. Embryo culture

Embryo culture is the sterile isolation and growth of an immature or mature embryo in vitro, with the goal of obtaining a viable plant. Embryo culture, the first in vitro culture method described in literature, is manly applied by plant breeders, especially in interspecific breeding programmes. In principle, there are two types of embryo culture: 1. Culture of immature embryos originating from unripe seeds, which is mainly used to avoid embryo abortion, 2. Culture of mature embryos derived from ripe seeds,which is used to eliminate the inhibition of seed germination.

The most important applications of embryo culture are:

1. Elimination of the (absolute) inhibition of seed germination.

2. Germination of seeds of obligtory parasites without the host.

3. Shortening the breeding cycle. By removing the seed coat and/or endosperm, seeds can germinate immediately.

4. Production of haploids. Haploid embryos are only viable by applying embryo culture.

5. Prevention of embryo abortion in early ripening stone fruits.

6. The prevention of embryo abortion as a result of incompatibility.

7. In Gramineae and Gymnospermae embryos are often used as starting material for micropropagation because they are juvenile and therefore very responsive.

2.2. Germination of orchid seeds.

Since in nature orchids live in a symbiotic relationship with fungi (mycorrhiza), it was originally thought that they could only germinate and develop in vitro in the presence of a fungus. In 1922 it was shown that

asymbiotic germination is possible on relative simple media containing minerals and sugars. At present most cultivated orchids and almost all wild orchids can be grown in vitro without a fungus.

Since the discovery of asymbiotic germination, numerous specific media have been developed for the many different genera and species. Very often complex mixtures are necessary to induce germination and seedling growth in vitro: banana homogenate, peptone, tryptone, coconut milk, yeast extract, etc.

The reasons to sow orchids in vitro can be summarized as follows:

I. The small size of the orchid seeds makes that they can easily be lost if sown in vivo.

2. The limiting food reserves in seeds make survival in vivo unlikely.

3. Germination and seedling development of orchids in nature are dependent on a symbiotic relationship. This relationship can relatively easily be simulated by a nutrient medium with special additives.

4. Sowing in vitro makes it possible to germinate immature orchid embryos, leading to a shortening of the breeding cycle.

5. When in a particular cross only a restricted number of seeds becomes available, in principle all of them can germinate and develop in vitro.

6. In vitro germination and seedling development are much faster as compared with in vivo since there is a conditioned environment, and no competition with fungi, bacteria, or other plants.

2.3. Micropropagation of orchids

If seeds from cultivated orchids (Cymbidium, Cattleya, etc.) are used (manly obtained from a cross and very heterozygous), the progeny will be extremely heterogeneous. To obtain an identical progeny cultivated orchids can only be propagated vegetatively which is a very slow process using in vitro techniques.

In 1960 a revolution took place in the micropropagation of orchids. In France Morel discovered that isolated shoot tips of orchids in vitro can be rejuvenated in vitro and form (regenerate)so-called protocorm-like bodies; these bodies are extremely similar to those already well known from orchid seed germination. Sometimes such a protocorm divides spontaneously, but usually this is brought about by cutting the protocorm into pices. When cutting is stopped, the shoot tip in each protocorm is allowed to develop a shoot with leaves and roots. In this way thousands of plants can be produced in one year. Cloning of orchids by meristem culture (also called mericloning) became the first commercial application of micripropagation. The method originally developed for Cymbidium, was later modified and used for Cattleya and many other orchid genera. Mericloning of orchids is now carried out on a large scale world wide.

Over the years a few other methods apart from meristem culture have been developed for micropropagation of orchids: young leaves, dormant buds, young inflorescences, callus, and young seedlings.

2.4. Production of disease-free plants

Internal infections caused by viruses, mycoplasmas, bacteria and fungi can be very difficult to eliminate. These pathogens are nearly always transferred by vegetative propagation, but they also can be transferred during generative propagation. Sometimes heat treatment is an effective way of inactivating some viruses, especially isometric viruses and mycoplasmas, heat treatment is often applied in fruit trees, sugar cane and cassave.

There are 5 available method of producing virus-free plants:

1. Heat treatment, 2. meristem culture, 3. heat treatment followed by meristem culture, 5. grafting of meristems on virus-free root stocks, also known as micro-grafting. Method 4 and 5 are applied in very special cases. To obtain virus-free meristems on a large scale, in vitro adventitious shoot formation on explants (e.g. in lily and hyacinth) may be induced, after which meristem culture is applied. If isolated meristems do not grow in vitro they can be temporarily micrografted on in vitro grown virus-free seedlings or rootstocks (e.g. in Citrus).

In France Morel and Martin had the brilliant idea of isolating in vitro the apical meristem of dahlias infected with virus, from which they were able to obtain virus-free plants. Their idea was based on the fact that the virus was unevenly distributed in the shoot of plants. After this discovery meristem culture has been applied in many vegetatively propagated plant species, especially in horticulural crops. Later meristem culture was also effectively applied to obtain bacteria- and fungus-free plants.

2.5. Micropropagation

The classical methods of in vivo vegetative propagation often fall short of that required (too slow, too difficult, or too expensive) or are completely impossible. In the last 15 years, since the discovery that plants can be cloned more rapidly in vitro than in vivo. knowledge concerning micropropagation has grown

quickly. Since this subject will be extensively treated in chapter 3, the reader is referred to this chapter.

2.6. Somaclonal variation

When natural genetic variation (e.g. in chimaeras) is observed in plants which have been regenerated in vitro (often as a result of genetic instability), then the term somaclonal variation is used. Many factors determine the chance of mutation and the mutation frequency during in vitro culture: the micropropagation method used, the type of tissue used, the starting material and the number of times subculturing. The possible use of somaclonal variation for crop improvement was extensivily examined, e.g. in potato, tomato and sugar cane. Somaclonal variation is particularly interesting in plants which show little variation in nature, or in which variation is difficult or impossible to induce. In a number of plant species resistance to a particular disease, drought or salt was obtained from cultured cells.

2.7. Test tube fertilization

In vitro fertilization is of particular importance in plant breeding if the incompatibility is present on the stigma or in the style. This technique is also applied, when the abscission of a flower is sometimes unavoidable, or for the production of haploids.

In vitro fertilization can be performed in 4 different ways:

1. Stigma fertilization: an emasculated flower is isolated in vitro and pollinated.

2. Placental pollination: placenta explants with unfertilized ovules are isolated and pollen grains are placed near the ovules.

3. Fertilization of isolated ovules without a placenta.

4. Fertilization on decapitated styles.

2.8. The production of haploids

Haploid cells or individuals are those in which the original chromosome number has been reduced by half. If the number of chromosomes of a haploid is doubled, either spontaneously or by induction (with the help of colchicine) a homozygous diploid individual is formed with 2 identical sets of chromosomes. Obviously plant breeders have searched intensively for haploids that have been arisen spontaneously or attempted to make them artificially. Since 1964 a new tool, in vitro culture of anthers, became available to induce haploids. Although in the plant (in vivo) mainly the female egg-cell or synergid is stimulated to grow without being fertilized, in vitro virtually only androgenesis takes place. In androgenesis the vegetative or generative nucleus of a pollen grain is stimulated to develop into a haploid individual without under going fertilization. The advantages of haploids are:

1. Haplid induction followed by chromosome doubling results in homozygosity in the quickest possible way, making breeding work much easier.

2. Homozygosity is very important for breeding of plantas which have a very long juvenile phase, like fruit trees, bulbous plants and forestry trees.

3. The production of pure F1-hibrids is possible as soon as homozygotic lines become available.

4. Induction of haploids in polyploid plantas results in much easier breeding at a lower ploidy level.

5. For the mutation breeder monohaploids have the advantage that recessive mutations are immediately discernable.

6. By haploid induction followed by chromosome doubling it is possible to obtain exclusively male plantas, e.g. in Asparagus officinalis.

2.9. Genetic manipulation

Until 1970 sexual reproduction was only possibility for recombination of genes in higher plants. Since the discovery of protoplast fusion in 1970, asexual ways of transmitting genetic material have been developed. Very essential for this development has been:

1. the production of protoplasts (cells without cell walls), their fusion and the regeneration of plants from (fused) protoplasts, 2. the discovery that bacteria are capable to transform a plant, if a plant cell becomes infected e.g. by Agrobacterium tumefaciens, then a part of the plasmid of these bacteria becomes built into the DNA of a plant.

Fusion of protoplasts of sometic cells of 2 plant species can lead to so-called somatic hybrids with characteristicas of both plants. When protoplasts fuse completely both the nuclear and cytoplasmic genetic information is transmitted from both partners to the somatic hybrid, in case of sexual reproduction the hereditary characteristics localized in the cytoplasm are usually only heridited from the mother. Sometimes there is partial transmission of genetic material when e.g. one of the protoplasts is irradiated (resulting in loss of chromosomes), or if spontaneous chromosomal elimination occurs. Also the formation of so-called cybrids is possible, in this case the nucleus and cytoplasm of one protoplast fuse with only the cytoplasm of another protoplast, the formation of cytoplasmic hybrids (called cybrization) is especially interesting with respect to the transmission of cytoplasmic male sterility, which mainly is located in the mitochondria.

Another interesting development in genetic manipulation is the transplantation and uptake of isolated nuclei, chromosomes, chromosome fragments, organelles (plasmids and mitochondria) and DNA. For instance it would be interesting to implant a piece of cromosome on which a gene for disease resistance is localized.

A very spectacular development in genetic manipulation is transformation with the aid of the bacteria Agrobacterium tumefaciens. The transfer of the manipulated plasmids of these bacteria leads to very efficient transmission of genetic information (DNA), and is frequently used with dicotyledons. Transmission of herbicide, virus, and insecticide resistance and even genes for flower colour (Petunia has recently been achieved with the aid of bacteria. In recent years particle gun bombardment has become and important tool for genetic manipulation.

Centainly there are quite a number of bottle-necks for genetic manipulation:

1. Regeneration techniques are often lacking, especially in shrubs and trees.

2. Often it is unknown which gene(s) is (are) responsible e.g. for resistance against disease, drought, salt, etc.

3. Isolation and cloning of genes through molecuar biological techniques are not yet available.

4. Polygenic determined characteristics are extremely difficult to transmit.

5. Tranformation techniques are unknown (e.g. transformation of monocotyledons by Agrobacterium tumefaciens).

6. Much more effort and research is required to find genetic information (genes) in nature, to explore it an to transmit it to our cultivated plants.

7. Transient expression of genes.

2.10. Miscellaneous applications

In phytopathology

1. In vitro culture of a parasite and host under controlled conditions with the elimination of other undesired parasites.

2. Utilization of in vitro culture to provide a better in sight into the behaviour of viruses in plant cells, and tissues.

3. As a tool in nematode research.

4. Studying tumour induction which may be achieved by Agrobacterium species, wound virus and crossing.

5. Transpor of disease-free plant material in vitro which is generally accepted as safe.

In plant breeding

1. The development of chimaeras in vitro by adventitious shoot formation or by mixing cell suspensions shich consisted of a mixture of cells.

2. Separation of chimaeras by adventious shoot formation.

3. Isolation of mutations. Often the mutated part of a plant is so small that taking a cutting in vivo is impossible, in that case shoot formation in the in vitro isolated mutated sector is then necessary.

4. Doubling the chromosome number in vitro can be easily brought about in vitro by the addition of colchicine, and sometimes by the induction of adventitious shoot formation.

5. Mutation induction in vitro is a very attractive method, especially in combination with adventitious shoot formation. Adventitious shoots usually originate from a single cell, resulting in so-called solid (complete) mutants.

6. Storage of plant material in vitro, especially for vegetatively propagated plants. Storage (gene banks) can be realized at (extreme) low temperature, disease-free, and under other conditions which limit growth. At this moment meristems and shoot tips are preferred for the storage of plant material in vitro because their genetic stability is high.

2.11. The biosynthesis of substances in vitro.

In analogy with micro-organisms attempts have successfully been made to obtain substances from root cultures and cell suspension cultures of higher plants, either through accumulation in the cells or by the release into the nutrient medium.

3. MICROPROPAGATION

3.1. Introduction

The most important application of in vitro culture is cloning, also called micropropagation or vegetative propagation. The aim of micropropagation is to create safe possibilities to clone higher plants disease-free, faster and less expensive way compared with in vivo techniques than in. In vitro cloning also offers possibilities to clone plants in cases where it is impossible in vivo. An additional advantage is that micropropagation enables the production of disease-free plants and there by also facilitates the so-called phytosanitary transport.

3.2. MICROPROPAGATION SYSTEMS

Several methods have been developed to propagate plants in vitro. Since the reliability of these methods differs markedly, as does their convenience of application. They are summarized below (Pierik, 1987, 1988).

Single-node and rooting of ensuing shoots

The most obvious, simple and secure method of cloning plants in vitro is single-node culture, resulting in the production of shoots and the regeneration of roots on these shoots. With typical rosette plants, this method is difficult to realize due to problems of infection when isolating in vitro. However, those plants forming an elongated stem with leaves and buds in the axils of these leaves, can be very easily propagated using this method. As soon as a bud or a shoot tip grows out into an elongated stem, the single nodes with dormant buds are subcultured to form an elongated stem again, and the apical shoot tips can be rooted directly. This systems was successfully aplied (Pierik, 1987, Pierik et al., 1988) to lilac (cultivars and root stocks) and various vegetables (tomato, pepino, cucumber and sweet pepper). With this system the rate of propagation is strongly dependent on the number of nodes (leaves) formed in vitro within a certain time period. This method is the most natural in vitro propagation method that exists. The method is also very safe, because the integrity of the plant (in particular meristems and buds) is not disturbed. It should be noted that in most cases the method can be successfully applied to herbaceous plants. When cloning of shrubs and trees is attempted with this method, in some cases serious problems with dormancy of buds and elongatin of shoots may arise.

Axillary braching

The second, slightly more artificial method is the induction of axillary braching in isolated shoot tips. In nature, apical dominance can be broken by removing the apical bud, resulting in a release of dormancy of the axillary buds, as soon as the apical bud is removed, the basipetal flow of auxin which normally keeps the axillary buds dormant, stops.

Removing or killing the apical bud also breaks apical dominance in vitro, resulting in axillary braching, but in most cases this is realized by the application of cytokinins, which are capable of antagonizing the auxin produced by the apical meristem.

As soon as axillary buds or shoots are formed, they can be separated from the original shoot tip. They can again be placed on a fresh medium with cytokinin to repeat axillary braching, etc. When enough shoots have been obtained, the process of axillary braching is stopped by omittig cytokinin, subsequently auxin is applied to promote rooting of the individual shoots in vitro or sometimes in vivo.

The axillary braching method is frequently applied, since it is relatively simple and quite safe. If no adventitious buds are formed, generally no mutations occur. In most cases regeneration of adventitious buds is a unwanted side-effect of excessive cytokinin levels in the medium during axillary branching.

In practice the axillary shoot method readily has become the most important propagation method in vitro because:

- Generally it is simpler than the other methods of propagation.

- The rate of propagation is relatively high.

- Genetic stability is usually preserved.

- The growth of the resultant plants is very good, due to rejuvenation and/or the lack of micro-organisms in the plants.

Regeneration of adventitious buds and roots

The third important method used to clone plants in vitro is called the adventitious bud/shoot technique. An explant (from leaf, petiole, stem, stalk, corm, etc.) without pre-existing buds or shoots is isolated in vitro. In most cases cells in the explant dedifferentiate and subsequently start to divide. Under the appropriate conditions, cell division can be directed in a very special way so that adventitious buds arise, which can develop into shoots. These shoots can be rooted or used to continue shoot formation. When adventitious bud formation can easily be induced in vitro, the method has many advantages. The number of plant species that can regenerate adventitious buds on excised explants is relatively small and often restricted to herbaceous plants, woody plants, particularly in the adult phase, are almos incapable of regenerating buds.

The greatest difficulty with the adventitious bud technique is that the chances of obtaining mutated plants are much higher than when the single-node or the axillary branching method is applied. This holds particularly true for (crypto) chimaeric plants and also for plants developing adventitious buds from single cell origin. The adventitious bud technique is quite successfully applied in the following plant species, which are relatively easy to regenerate, lily, Saintpaulia ionantha, hyacinth, Begonia, Achimenes, Streptocarpus, Cichorium intybus, and chrysanthemum. The auxin and cytokinin requirement for adventitious budding can be very different for various plant species and even cultivars. A small number of plants such as lily and hyacinth react positively to auxin application, whereas many species require cytokinin to form adventitious buds. Others require both cytokinin and auxin. In many

species a relatively high cytokinin level and a low auxin level promote adventitious budding.

Regeneration of plants from callus, single-cells and protoplasts

Callus is a strongly dividing and more or less undifferentiated tissue. It can be obtained by isolating tissues, organs, embryos single cells or protoplastys in vitro, differentiated tissues generally first undergo dedifferentiation before cell division starts. Under appropriate conditions, callus can be subcultured in vitro without organ or somatic embryo formation. However, it is also capable of regenerating somatic embryos or adventitious buds if conditions are changed in the appropiate way.

Although many research workers claim that cloning of higher plants through callus is a very attractive method other researchers have found just the opposite. The greatest difficulty with callus, and also with single cell and protoplasts cultures, is genetic instability. Plants originating from protoplasts, single cells and callus very often are mutated. Another handicap of callus culture is that during prolonged subculture the organ (embryo)-forming capacity may be lost. Only in a few plant species, such as Anthurium andraeanum and coffee, is callus cuture in most cases a satisfactory and rather safe method, applicable by commercial tissue culture companies.

4. APPLICATION OF MICROPROPAGATION

It is especially notable in horticulture that people have quickly responded to the results obtained from research on micropropagation. Since there are no accurate and specified statistics on the extent to which cloning in vitro is being used commercially, first of all an analysis will be given of the application of micropropagation in the Dutch horticultural industry. The Netherlands was chosen because detailed information was available (Pierik, 1990b, 1991, 1991b, 1993) and because this country dominates the world export market of cut flowers, pot plants and bulbous and cormous plants. A recent study (Pierik, 1991) showed that the Netherlands has a total of 78 commercial tissue culture laboratories with a production of 100 million plants (Table 1)

An analysis was done (Pierik, 1990, 1990b) of commercial micropropagation in 15 West European countries. In 1988 Western Europe, had a total of 248 commercial tissue culture laboratories. The total production for 1988 in Western Europe is summarized in Table 2, which shows that ornamental plant species (157 million or 74% of the total propagation) dominate micropropagation in this part of the world. The most frequently cloned ornamental plants are: Ficus, syngonium, Spathiphyllum, Gerbera, Rosa, Philodendron-Saintpaulia, Nephrolepis, Cordyline, Anthurium, Calathea, Cymbidium, Dieffenbachia and Rhododendron.

TABLE 1. Micropropagation of ornamentals in the Netherlands in 1990 (Pierik 1991a). Numbers of plants are given in millions.

Plants produced per laboratory	Commercial laboratories	The most important pot plants,	
Less than 0.1	35	Nephrolepis	17.8
0.1-1.0	25	Saintpaulia	2.7
1.0-1.5	12	Spathiphyllum	5.4
More than 5.0	6	Syngonium	3.7
		Ficus	3.3
Total	78	Anthurium scherz.	4.2
The most important cut flowers		Other important ornamentals	
Gerbera	15.1	Lilium	23.2
Aster	1.6	Cymbidium	1.7
Anthurium andraeanum	1.5	Other orchids	1.9

TABLE 2. Micropropagation in Western Europe in 1988. Plant numbers are given in millions (Pierik, 1990c).

Categories		Categories	
1. Pot plants	92.34	8. Perennial garden plants	2.98
2. Cut flowers	37.84	9. Agricultural crops	2.42
3. Fruit trees	19.43	10. Miscellaneous ornamentals	1.94
4. Ornamental bulbs and corms	13.16	11. Vegetables	1.38
5. Small fruits	9.35	12. Trees (forestry)	1.29
6. Orchids	5.29	13. Herbs	0.03
7. Ornamental trees and shrubs	3.89	14. Not specified	21.13
Total category 1-14	212.47		

Several authors in a new handbook on micropropagation (Debergh and Zimmerman, 1991) gave a description of micropropagation in the whole world. The production figures from all parts of the world are summarized in Table 3.

5. ADVANTAGES OF MICROPROPAGATION

Micropropagation has the following advantages (Pierik, 1987, 1988, 1993).

TABLE 3. Commercial micropropagation in the whole world in 1989 (estimated by R.L.M., Pierik)

	Millions of plants produced
The Netherlands	80
France	46
Italy	34
Other West European countries	60
East European countries	34
Israel	6
Africa	?
Middle and South America	?
Australia and New Zealand	46
Asia	92
United States of America	115
Total	513

- When classical methods of in vivo vegetative propagation prove to be inadequate, in vitro cloning may be an important tool in speeding up propagation.

- Adult plant material, which often cannot be cloned in vivo, can sometimes be rejuvenated in vitro and subsequently cloned. (see chapter 8).

- Growth of in vitro propagated plants is often stronger than of those cloned in vivo; this is mainly due to rejuvenation and/or the fact that they are disease-free.

- When the existing methods of cloning are too slow or too complicated to be profitable, in vitro cloning can be applied to produce large numbers of plants more quickly and at a competitive price.

- By in vitro cloning expensive methods such as grafting or budding on a root-stock can be rendered obsolete in a number of cases.

- In vitro cloning enables, the uncovering of chimaeras and the isolation and cloning of spontaneous or induced mutants. Mutation induction and regeneration of adventitious buds in vitro enables to obtain solid mutants. The reason for this is that adventitious plants often find their origin in one cell.

- In contrast to in vivo propagation, in vitro cloning of plants can be continued year round and so become independent of the seasons.

- In vitro storage and cloning facilitates the creation fo gene banks (preservation of valuable plant material) and the storage of plants under pathogen-free conditions on a relatively small surface. By use of low temperature storage and freezing, time spent on cloning as well as the space required can be drastically decreased. Low temperature storage also makes it possible to stagger production more effectively than under in vivo conditions.

- In vitro cloning facilitates the production of disease-free plants and thereby the phytosanitary transport from country to country.

- Greenhouses are expensive and energy prices are high. These costs can be reduced by in vitro cloning as very few stock plants are required as starting material and much less greenhouse space is required for making cuttings.

- For plant breeders propagation in vitro facilitates cloning of parent plants as starting material for hybrid seed production. Another argument for plant breeders to apply in vitro cloning is: a new cultivar can be cloned much faster and consequently sold earlier than when classical propagation techniques are applied.

- In vitro cloning enables genetic manipulation (engineering), which would be impossible if there were no methods available for regenerating protoplasts, cells and tissues.

6. REQUIREMENTS OF MICROPROPAGATION

When plants are micropropagated, the following requirements should be met (Pierik, 1987, 1988):

- Genetic stability (no mutations).

- Thorough selection of plant material, in particular freedom from pathogens.

- Reversion from the adult to the juvenile phase should be possible, particularly when propagating shrubs and trees.

- Retention of the regenerative ability.

- Economic justification vis-a-vis the production of the same plant in vivo.

- The transfer from test tube to soil should not be too complicated and must be carried out without too many losses.

- In vitro propagation should not be too complicated, other wise it will be rejected in practice.

- Monoclonal plantations are only acceptable under strictly controlled conditions (glasshouses); in forestry e.g. multiclonal plantations should be realized.

7. NEW DEVELOPMENTS

7.1. Ex vitro rooting in Rockwool microplugs

In recent years inert synthetic supports for micropropagated shoots have been developed e.g. by Baumgartner Papier in Lausanne (Sorbarods) and by Milcap France (substrates 'Milcap', lumps and plugs). These supports are useful (Roberts and Smith, 1990), but the cost price is relatively high. However, the cost

price of Rockwool supports is much lower (U.S. $ 0.025).

In 1988, Grodan-Rockwool in cooperation with the Department of Horticulture, Wageningen Agricultural University in the Netherlands (Pierik, 1990, 1991, 1991b, 1993), introduced Rockwool microplugs which are specially developed for rooting of micropropagated shoots in vivo. Rockwool, well known as a growing medium for many horticultural crops (3000 ha in the Netherlands), is at present mainly used for Gerbera and Anthurium andraeanum microcuttings. The microplug of mineral wool has now also been introduced in the Netherlands, especially to reduce labour and material costs and to increase the quality of the micropropagated plants.

The following are some of the unique properties of Rockwool:

1. It replaces soil so that export of plants becomes possible to those countries who refuse soil as part of their plant protection regulations.

2. It has the capacity to achieve a homogeneous water/air balance, including an optimal distribution of water, nutrients and air in the root environment.

3. Roots can penetrate the well-drained substrate very easily and shoots can easily be transferred to other Rockwool blocks of larger size or to soil to continue further growth. Rockwool enables transport without damaging the root systems and quick regrowth after transplantation.

4. It is sterile, eliminating the need for soil steaming or chemical sterilization.

5. It allows the regeneration of normal roots versus abnormal root formation in agar media. The plugs have a special fiber structure to promote rooting.

6. It is almost chemically rather inert, containing no plant nutrients, or contaminants of organic origin normally present in agar.

7. Tray systems for microplugs facilitate transport.

However, there are a number of special requirements to be kept in mind for rooting:

1. Plugs should be fully saturated with a weak nutrient solution (Electrical conductivity 0.5-1.0 μS/cm) before shoot cuttings are inserted. Plugs should be allowed to leach out before the cuttings are inserted.

2. Auxin requirement for cuttings is strongly dependent on the plant species and cultivar, and also on the hormonal composition of the culture medium just before shoot cuttings are taken.

3. Shoots should be washed to remove sugar from the culture medium, otherwise infections occur easily.

4. Since Rockwool initially has a slight basic reaction, the nutrient solution (dependent on the plant species) should be used at a pH of 5.2.

5. Relative humidity (90-95%) should be carefully controlled, especially during the first 1-2 weeks after inserting the shoot cuttings. Acclimatization should take place by gradually decreasing the relative humidity.

6. Regular application of water and nutrients is required, depending on water loss and location in the greenhouse.

7. The quality and size of the microshoots is more critical in Rockwool plugs than in agar media.

8. The greenhouse climate (temperature and relative humidity) needs to be controlled carefully during rooting and acclimatization in Rockwool. This has been shown for Gerberas.

9. During the first week after transplanting rooted plants in plugs to soil, care must be taken that plants do not dry out, since soil easily attracks water from the relatively small plugs.

7.2. Agar quality

Agar is a hydrophilic colloid extracted from seaweeds, belonging to the Rhodophyceae (Pierik, 1991, 1991b). It consists of 2 fractions (agarose and agaropecting) and is the classical gelling agent used in plant tissue culture (Armisen and Galatas, 1987). Agar is purified by the manufacturers and should contain no toxic compounds. The ash content (inorganic salts) of agar varies from 2.5-5.0% (w/w). The ash content of agarose is much lower than that of agaropectin due to the absence of ionic groups.

Analyses of agars have shown that they contain many organic and inorganic impurities (Kohlenbach and Wernicke, 1978; Debergh, 1983; Pierik, 1987; Scherer et al., 1988; Kordan, 1988). It is not known which undesired contaminants should be removed to obtain a qualified agar with an acceptable ash content. Toxicity is certainly dependent on the element under consideration. Debergh (1983) demonstrated that inorganic impurities introduced with the agar are responsible for significant differences in the concentration of several ions in addition to the ions added with the mineral nutrition. Nothing about the nature of organic contaminants can be found in literature.

Remarkable effects of various agar brands have been described in the literature. The following processes were strongly influenced by the agar brand: pollen germination and pollen tube growth (Kordan, 1988), differentiation of tracheary elements (Roberts et al., 1984), anther regeneration (Kohlenbach and Wernicke, 1978), regeneration of sugar cane (anders et al., 1988) and

Kalanchoë (Hauser et al., 1988), and shoot proliferation of apple and pear (Singha, 1984).

Quite recently the effect of agar brands on adventitious shoot and root regeneration, bulblet formation, and axillary shoot formation has been studied. Interactions were found between the agar performance and the plant species, the explant type, the cultivar, and the cultural conditions. The agar quality effected all developmental processes, the regeneration of adventitious shoots and roots and sprouting of axillary shoots being the most sensitive. In well established cultures the agar sensitivity was reduced. The degree of chlorosis and necrosis of woody plants could also be related to agar quality. From wxperiments with 14 plant species it appeared that agars can be identified, which In general have a good performance (Scholten et al., 1994).

7.3. A new automatization system with membrane capsules

Recently a Dutch company in Wageningen, called PermX Multiplant (Pierik, 1991b), introduced a new revolutionary production technlque which enables automatization of micropropagation. The most important principle of the PermX machine is that presterilized membrane capsules (see section 9.4) are automatically filled with sterilized nutrient media. The PermX technology reduces costs because many activities applied in conventional tissue culture laboratories are now automated. The inoculation of e.g. scale explants from lily and sealing (closing) of the membrane capsules is completely automated. The cutting of explants is still done by hand. The advantages of the machine are summarized below.

1. Reduces costs.
2. Many activities are now automated.
3. More efficient production process.
4. Better plant quality and viability.
5. Flexibility for both producer and consumer.
6. Application of membrane capsules.
7. Optimizes quantity and composition of medium.
8. Individualized cultivation method.

7.4. Micropropagation in membrane capsules

Culture of shoots and explants in the new automatization system described in section 7.3 is carried out in membrane capsules. Shoots or explants are cultured on agar media in a modified atmosphere through membrane diffusion in polyethylene bags. The new container consists of a membrane capsule, allowing a maximum of gas diffusión (exchange) with the environment (Gerrits, 1990). The advantages of membrane capsules for plant tissue culture and micropropagation are summarized below (Pierik, 1991b).

1. Higher multiplication rates can be obtained.
2. The quality of the microplants increases.
3. Better plant vigour.
4. No competition between plants.
5. Absence of risk of infection by microorganisms.
6. Membranes can be chosen with different gas permeability.
7. Possibilities of extended plant development.
8. Aseptic rooting in Rockwool plugs is possible.
9. Early acclimatization takes place.
10. Membranes are impermeable to water.
11. Membranes are an absolute barrier for microorganisms pene trating into other bags.
12. The diffusion capacity of the membrane is controllable.
13. Storage density in the culture rooms increases considerably.
14. The production capacity can be increased.

8. REJUVENATION

Maturation is the major problem preventing a wider application of tissue culture technology among woody species, especially trees. Successes with a small number of trees has been achieved mainly with special starting material, certain pretreatments and manipulations (Pierik, 1987, 1991b, 1990d). The transition from juvenile to adult, which is localized in the meristems, has a negative effect on propagation. The characteristics of mature woody plants and the possibilities to micropropagate adult trees and shrubs are summarized below.

Mature woody plants show:

1. A reduced growth rate.
2. A reduced or total lack of rooting ability.
3. Plagiotropy.
4. Ability to flower.

Ways for micropropagation adult shrubs and/or trees:

1. Using juvenile buds and/or epicormic buds (stump sprouts) at the base of the tree.
2. Etiolation, ring barking or girdling.
3. Forcing spaeroblast.
4. Use of root suckers.

5. Isolation of shoots from lignotubers, water shoots, or reiterated shoots.

6. Special (pre.) treatments to rejuvenate or to reinvigorate:

- Hormone treatment, especially cytokinins.

- Selection of vigorously growing buds.

- Isolation of meristems or shoots.

- Severe hedging, pruning, cutting back, stool beds or stool layering.

7. Repeated subculture in vivo or in vitro of (micro-) cuttings or shoots.

8. (Micro) grafting of adult meristems/shoots on juvenile rootstocks.

9. Adventitious shoot and/or somatic embryo formation.

10. Asexual reproduction of plants which show apomictic or nucellar embryony.

11. Co-culture of juvenile and adult shoots in one container.

12. Use of nucellar tissues as centres (islands) of juvenility.

9. PRODUCTION COSTS

A starting tissue culture laboratory needs an investment of about U.S. $ 300,000. The first five years are crucial. In this period the investment will have to be doubled, but also the break-even point must be reached. A larger part of the production must exist of cultivars in large quantities at reasonable prices. These plants will lower the unit price and take a large part in the overhead costs (Pierik and Scholten, 1994).

Depending on the species and cultivar the cost price of tissue culture plants in the Netherlands is 15-30 U.S. $ cts. Standaert-De Metsenaere (1991) gave a general stucture of the costs. The costs of labour are 60-80% of the total costs. In smaller and specialized laboratories the cost of labour account for a relatively large part of the costs. The costs of labour (as an example 65%) can be divided in production (32.5%), R&D (6.5%), and other activities like media preparation, administration, supervision (26%). The media costs are 7%. Equipment and laboratory costs account for 17%. The remaining 11% consists of costs for office, representation, electricity, etc.

In vitro plants have to compete with in vivo propagated plants and seeds. The price for extra quality is generally not paid by the customers. Therefore, in general the profits are low and consequently a low budget is available for R&D, diversification of products, automatization and development of specific features of tissue culture. The costs can be lowered by a few percent by efficient use of new equipment for culture rooms, plant density, etc. By a drastic reduction of labour costs and development of more efficient propagation techniques, the marketshare of micropropagated plants can expand.

10 DEMANDS OF CUSTOMERS

Major demands of the customers, where micropropagated plants concerned, are:

Product attributes

1. High quality plantlets; homogeneity and uniformity of the plants.

2. Good plant form and not too bushy.

3. Easy to acclimatize and to bring into production.

4. No mutations.

5. A low percentage of infections when transferred to soil.

6. Reasonable and competitive prices.

Services

1. Delivery on time.

2. Periodical delivery of large quantities.

3. Culture and acclimatiztion advice.

4. Adquisition from more than one source to spread risks.

11. PROBLEMS IN THE WESTERN EUROPEAN TISSUE CULTURE LABORATORIES

Problems encountered in micropropagation in Western Europe can be summarized as follows:

Technical

1. Year-around production.

2. Lack of acclimatization ex vitro.

3. External and especially internal infections.

4. Induction of dormancy in bulbous crops.

5. Limited possibilities to automatize.

6. Genetic instability in callus systems.

7. Unwanted after effects (e.g. branching).

8. Each cultivar and species requires different media, etc.

9. Rejuvenation in shrubs and trees.

10. Excretion of toxic substances.

11. Problems with scaling up.

12. Problems in production management.

13. No diversification of product.

Organizational, especially peaks in labour demands

1. Acquisition of temporary labour.

2. Contracting out during production peaks.

3. Organization of labour shifts.

4. Staggering of labour throughout the year.

5. Choosing complementary crops.

6. Cold storage.

7. Automatization of the production processes.

Economical

1. Financing and setting up a laboratory.

2. No intervention by third parties which increase the cost of production.

3. Difficulties in lowering the cost of research, overhead and labour.

4. Profits do not come from low-priced standard items.

5. First profits come after one year of production.

6. No diversification of products.

Marketing position

1. Strong competition from countries with low labour costs.

2. Overproduction of a number of easy-to-clone popular crops.

3. Summer dumping.

Marketing

1. Economic justification vis-a-vis the production costs of the same plant in vivo.

2. No access to and knowledge of foreign markets.

3. At present completely dependant on the production centres of ornamental crops, because most products are marketed in the shpere of ornamental horticulture.

4. Difficulties in finding new products and markets.

5. Lack of marketing exercise.

6. Language and communication problems.

12. PRODUCION TRENDS IN THE NETHERLANDS

The rate of increase in the number of commercial laboratories (Pierik, 1991) and also in the production of micropropagated plants is slowing down, especially in the Netherlands, but also in other West European countries. In the period 1992-1993 a few larger Dutch tissue cultures laboratories discontinued their production. This is primarily due to competition on the West European market, where prices of a few standard crops (such as lilies, gerbera, Nephrolepis and Saintpaulia) have been strongly decreased in recent years (Pierik and Scholten 1994). Since Eastern European countries (e.g. Poland, Bulgaria, etc.) and a number of the third world countries (e.g. India, Thailand, Indonesia, Singapore, etc.) have entered the West European market (Pierik, 1990b, 1991), the price fall is complete which is due to low labour cost in competitive countries.

13. CONCLUSIONS

Since only 1-3% of the total clonal propagation in the Netherlands is by micropropagation, it can be concluded that micropropagation is still in his infancy (Pierik and Scholten, 1994). A greater increase in micropropagation will occur when advance production techniques (e.g. synthetic seeds, bioreactors) are developed, when mechanization is introduced (e.g. robots, etc.) and when plant material from trees can efficiently be rejuvenated. We expect that a special and large market will be opened when tree micropropagation is possible on a large scale.

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